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EVALUATION OF THE VIRULENCE AND IMMUNOGENICITY OF VACCINE STRAINS OF SALMONELLA AND SHIGELLA BY DETERMINING THE MEAN (50%) DOSES [LD_{50}]

[Following is the translation of an article by Ye. N. Milikova and I. G. Vasilyeva, State Control Institute for Medical Biological Preparations imeni Tarasevicha, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology), #1, 1963, pages 92-98. It was submitted on 4 Oct 1961. Translation performed by Sp/7 Charles T. Ostertag Jr.]

As is known, in tests on laboratory animals, a calculation of the doses of the agents being tested that cause a 50% effect (ED_{50}) makes it possible to more correctly evaluate one or the other biological property of the preparation (Reed and Muench, 1938; Pomorskiy, 1935).

Troitskiy and Kovaleva, Gekker, Shvartsman, Kovalevskaya, Melikova, Leonteyeva and others have studied and recommended for putting into practice methods for the quantitative evaluation of the immunogenic properties of vaccines against intestinal infections by means of determining the minimum immunizing dose and the index of effectiveness.

However, according to regulated methods until now the immunogenicity of both typhoid-paratyphoid and dysenteric strains is determined according to the percentage of survival following infection with a dose that causes 100% death (Dcl). The virulence of salmonella and shigella strains is also evaluated by the Dcl. When using nonpedigreed mice the lethal dose of the strains, depending on the individual sensitivity of the animals, fluctuates sharply from test to test. This will often lead to the erroneous conclusions of Leontyev.

From what has been said it is apparent that it is necessary to introduce into practice in the vaccine industry, modern methods for determining the virulence and immunogenicity by means of calculating the mean 50% doses. With the aim of establishing the minimally acceptable quantities of these values for the vaccine strains of salmonella and shigella and introducing the appropriate changes in the instructions we conducted special investigations.

This communication presents the characteristics of virulence and immunogenicity of reference strains of the causative agents of the intestinal infections based on the LD_{50}, the minimal immunizing dose and the index of effectiveness.

The investigations included all the vaccine strains of typhoid, paratyphoid A and B and Flexner's and Sonne dysentery bacteria, used at the present time in industrial institutes for the preparation of vaccines against intestinal infections. In order to determine to what extent these reference industrial strains may reflect the level of virulence and immunogenicity inherent to certain species of microbes, we took freshly isolated and museum (stock) strains for a comparison. All told 45 strains were studied.

We conducted the determination of virulence and immunogenicity on nonpedigreed white mice, weighing 14-16 g and of a mixed group. The studies of strains of one species were conducted in a single test on animals that were supplied at the same time. The tests were repeated several times. For determining the LD_{50} the mice received the following multiple doses of culture intraperitoneally: For typhoid, paratyphoid B and Sonne dysentery cultures -- 200 - 100 - 50 - 25- million; for paratyphoid A and Flexner's dysentery -- 1 billion - 500 - 250 and 125 million of microbes. Five to six animals were taken for each dose. The cultures were introduced in a volume of 0.5 ml in a physiological solution. The tests were considered over a period of three days. For checking the virulence we used 20-hour cultures washed off from the agar. Typhoid and paratyphoid strains were incubated on Hottinger agar (1.5% concentration) with a pH=7.2, dysentery strains -- on the same agar with a pH=7.6. Preliminarily the cultural and serological properties and the antigenic structure of the strains were checked.

It must be noted in particular that throughout the entire work, for repeated tests we always used the 3-4th generation of the cultures after desiccation.

Heated vaccines from a 24-hour culture of the strains were prepared for checking the immunogenicity. The mice were immunized subcutaneously. For each strain four groups of mice were taken in conformity with the four doses of vaccine. There were 6-10 animals in each group. When checking the typhoid, paratyphoid and Sonne dysentery strains a single immunization was performed with 100, 50, 25 and 12.5 (and lower) million microbial cells in 0.5 ml of physiological solution. When checking paratyphoid A and Flexner's dysentery (type c) strains the mice were immunized twice, with a one week interval, with 200, 100, 50 and 25 million microbes. In 10 days following immunization the animals were infected by the intraperitoneal administration of 2.5--3 LD_{50} of a heterological strain of the same species. Calculation of the death of the animals was carried out over a period of three days.

For the purpose of calculating the regularity of the conditions of the test and the reliability of the data obtained when checking the immunogenicity of typhoid strains, the test included a standard of immunogenicity of the State Control Institute imeni Tarasevicha. This is a dry, heated divaccine (typhoid-paratyphoid B).

The results obtained in the testing of the virulence and immunogenicity were processed by the statistical method, calculating the median lethal (LD_{50}) and minimal immunizing doses. Considering that in industrial practice it is necessary to use the simplest and most practicable method for calculating the LD_{50} and the ED_{50}, we used the simplified formula of Reed and Muench (Troitskiy and Kovaleva).

In processing the data obtained with various strains in a number of subsequent tests, the average indices and their errors were calculated. These make it possible to determine the minimal requirements for the desired values (Kaminskiy). As the minimal requirement for determining the LD_{50} and the immunizing dose we computed the maximum permissible quantity of these values, established from the following calculation: The average plus the threefold error of the average (M + 3m). In determining the minimal requirement in respect to the index of effectiveness the minimal permissible value was calculated: The average minus the threefold error of the average (M - 3m). An evaluation of the immunogenicity of the strains based on the index of immunogenicity (II -- the relationship of the IE of the vaccine and the test strain to the IE of standard vaccine) was not conducted.

The data obtained when studying the virulence of strains of salmonella and shigella are summarized in table 1.

In 15 determinations the LD_{50} of industrial typhoid strains was found within the limits of 23-56 million. The actual value of it in vaccine strains constituted 39 million and the error of the average equaled 2.7 million. These quantities made it possible to establish the minimal permissible value of the LD_{50} for typhoid vaccine strains in limits up to 48 million (the figures in the last column of table 1, as well as in subsequent tables, are rounded off to whole numbers). Besides the 3 vaccine strains of the causative agents of typhoid, 5 stock strains were studied. Unconditionally the lethal dose in them(Dcl) equaled 100-125 million, and the average was found within the limits of 23-100 million. The minimal permissible value of the latter after the appropriate calculations comprised 78 million. Thus, in spite of the fact that all the strains, based on existing instructions, satisfied the requirements and had approximately the same Dcl value, they differed significantly according to the level of virulence when determining the value of the LD_{50}.

For reference vaccine paratyphoid B strains the LD_{50} fluctuated within the limits of 17.5 - 53 million. Its minimal permissible value can be considered as 50 million. For the remaining paratyphoid B strains this value equaled 64 million.

In Flexner dysentery strains (type c) the LD_{50} in 10 tests turned out to be equal to 200-310 million and its minimal value can be considered

as 275 million. In strains of the causative agent of Sonne dysentery the stated dose fluctuated within the limits of 25 -- 31.2 million, and its highest value, determined by statistical calculation, emanating from the average and its error, comprised 30 million.

We had only one reference strain of the paratyphoid A causative agent at our disposal, therefore we did not imagine it possible to conduct a statistical processing of the results of its verification. However, the data obtained made it possible to consider it suitable to use those paratyphoid A strains, the LD_{50} of which did not exceed 275 microbes, for the preparation of vaccine.

By carrying out a systematic parallel control of the antigenic structure of the cultures it was established that in the typhoid strains the value of the LD_{50} could change, depending on the changes in the antigenic structure of the strains, which is often the result of their prolonged storage and frequent reseedings on usual nutrient media.

Thus from table 2 it follows that the values of the LD_{50} of the Ty_2 4446 strain, in which d and Vi antigens were detected on glass with monoreceptor sera, equaled 33-40.5 million. In this same strain after prolonged storage and one reseeding the value of this dose approximately doubled (73-107 million). Simultaneously, during the determination of the antigenic structure it was possible to detect along with the d and Vi antigens a small amount of antigen 9.

It was also shown by the investigations that the virulence of typhoid and paratyphoid B strains depended on the age of the culture. This data is summarized in table 3.

In the Ty_2 4446 strain with 20-hour growth the value of the LD_{50} equaled 56 million, in the same strain of a 6-hour growth -- 12.5 million. In paratyphoid B strain No. 50602 the corresponding values were 53 and 10 million.

Consequently, virulence, as any other biological property, depends on the condition of the culture, its antigenic structure, age, conditions of cultivation and storage. All this again confirms that it is necessary to give particular attention to the standardization of all links of the biological test.

Paratyphoid A and Flexner's dysentery strains possess a high virulence for white mice. When controlling the immunogenicity of vaccines against intestinal infections in non-susceptable animals there is quite a bit of importance in the value of the resolving dose of the test-culture. Usually in order to obtain a lowering of the value of the resolving dose, they make use of infection with the culture in semiliquid agar. In connection with this, during the determination of the LD_{50} of paratyphoid A and Flexner's dysentery strains we used cultures in 0.4% agar.

As is apparent from table 4, under these conditions the value of the LD_{50} for paratyphoid A strains comprised from 7.6 to 13.6 million, and for Flexner's dysentery (type c) -- from 12.5 to 21.5 million. The maximum permissible values of the doses for these strains equaled 14 and 20 million respectively.

Determination of the minimal requirements for immunogenicity of strains of salmonella and shigella by means of determining the mean (50%) immunizing dose, the results of which are summarized in table 5, showed that in typhoid vaccine strains it fluctuated from 12.5 to 36 million, and in all the remaining typhoid strains from 12.5 to 45 million. The permissible value of the mean immunizing dose, that is, the minimum requirement for typhoid strains, can be considered as 31 million.

For paratyphoid B vaccine strains, the mean immunizing dose was found within the limits of 25 to 60 million. The minimum requirement was the value in 60 million, and for the remaining paratyphoid B strains -- 70 million.

In evaluating the immunogenicity of paratyphoid A strains based on the value of the mean immunizing dose, it turned out that the minimum requirement was a dose equal to 118 million, and for Flexner's dysentery (type c) -- 105 million. The mean immunizing dose of Sonne strains fluctuated from 25 up to 37.5 million and the maximum permissible value comprised 27 million.

The minimal requirements for immunogenicity of strains of typhoid and paratyphoid B bacteria in a determination according to the index of effectiveness are presented in table 6.

The data obtained shows that the value of the index of effectiveness was found in direct dependence on the dose of the vaccine that was selected for immunization. Thus, for typhoid strains during immunization with a dose of 25 million the index was 2, during immunization with 100 million -- 3.5; for paratyphoid B strains with a dose of 50 million the index equaled 2, with a dose of 200 million -- 3.

In dysentery strains during immunization with a dose equal to 50 million the index fluctuated within the limits of 1 -- 1.6, with a dose of 400 million -- within the limits of 2.3 -- 4.5. This testifies to the fact that in determining the index only the use of large doses of vaccine makes it possible to expose the immunogenic activity of the strains.

Conclusions

1. On the basis of the investigations carried out it can be assumed that the following requirements are minimum for the virulence of vaccine strains of salmonella and shigella: The LD_{50} of typhoid, paratyphoid B and Sonne

dysentery should not exceed 50 million and Flexner's dysentery c and paratyphoid A -- 275 million during infection in a physiological solution. During infection in 0.4% agar the LD_{50} of paratyphoid A and Flexner's dysentery c strains should not exceed 20 million.

2. The median (50%) immunizing dose during infection of 2.5 - 3 LD_{50} of a test-culture in typhoid and Sonne dysentery strains with a single immunization should not exceed 30 million, and in paratyphoid B strains - 60 million. In Flexner's dysentery c and paratyphoid A with a double immunization and infection of 2.5 - 3 LD_{50} of a test-culture it should not exceed 125 million. The index of effectiveness for typhoid, and Sonne dysentery strains with a single immunizing dose of 100 million whould be no lower than 3, and for paratyphoid B strains -- no lower than 2.5. For Flexner's dysentery and paratyphoid A strains with a double immunization with a dose equal to 200 million the index should not be lower than 3.

3. Cultures of typhoid and paratyphoid B strains of a 6-hour growth possess a higher virulence than 20-hour cultures.

4. The virulence of typhoid strains depends on the relationship of the antigens in the culture.

5. In checking the virulence and immunogenicity of salmonella and shigella strains it is necessary to pay particular attention to standardization of the conditions of cultivation and storage, and also the testing of the strains.

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Table 1

Minimal requirements for virulence of strains of salmonella and shigella based on the determination of the LD_{50}

Group of strains	Number of strains	No. of tests	LD_{50} value (in mil.)		Average and its error (M ± m)	Minimal requirement for LD_{50} (in mil.)
			from	to		
Typhoid	Vaccine-3	15	23	56	39±2.7	48
	Total - 8	8	23	100	50±9.3	78
Paratyphoid B	Vaccine-2	14	17.5	53	26±7.9	50
	Total - 8	21	21	85	46.7±5.6	64
Dysentery Flexner c	4	10	200	310	246±10	276
Dysentery Sonne	2	7	25	31.2	27±0.53	30

Table 2

Relationship of the antigens in a typhoid culture and virulence

Strain	Antigenic structure (agglutination reaction with monoreceptor sera on glass)	LD_{50} value (in millions)		
		1st test	2nd test	3rd test
Ty_2 4446	Vi ++ ++	33	31	40.5
Third generation	d ++ ++			
Ty_2 4446, prolonged passage on agar	Vi +++ d ++ ++ 9 ++	73	89	107

Table 3

Comparison of virulence of typhoid and paratyphoid B strains of 6- and 20-hour growth

Strain	Period of cultivation (in hours)	No. of tests	LD_{50} value (in mil.)		Average and its error (M ± m)	Minimal requirement for LD_{50} (in mil.)
			from	to		
Typhoid Ty_2 4446	6	9	8.1	16.6	10.4±0.8	12.5
	20	10	21	56	36±7.9	56
Paratyphoid No 50602	6	6	7.1	8.2	7.6±0.5	10
	20	6	21	53	23±10.2	53

Table 4

Minimal requirement for virulence of paratyphoid A and Flexner's dysentery strains following infection in 0.4% agar

Strains	Number of tests	LD_{50} value (in millions)		Average and its error (M ± m)	Minimal requirement for the value of the LD_{50} (in millions)
		from	to		
[illegible] typhoid A	8	7.6	13.6	10.8±0.8	14
Flexner's dysentery	6	12.5	21.5	16±1.5	20

Table 5

Minimal requirement for immunogenicity of strains of salmonella and shigella based on a 50% immunizing dose

Group of strains	Number of strains	No. of tests	Value (in mil.) of the mean immunizing dose		Average and its error (M ± m)	Minimal requirement (in millions) for the mean immunizing dose
			from	to		
Typhoid	Vaccine-3	6	12.5	36	21±3.6	31
	All told-8	16	12.5	45	22±2.7	31
Paratyphoid B	Vaccine-2	4	23	60	43±5.8	60
	All told-9	18	18	100	52±6.2	70
Paratyphoid A	2	9	50	100	78±13.3	118
Flexner's dysentery c	4	10	79	113	93±3.6	105
Sonne dysentery	2	10	25	37.5	21±1.6	27

Table 6

Minimal requirement for immunogenicity of strains of salmonella and shigella during during determination according to the index of effectiveness (IE)

Strains	Number of strains	Minimal permissible values of the index of effectiveness during immunization with various doses of vaccine (in mil.)					Note
		25	50	100	200	400	
Typhoid	9	2	2.5	3.5			Statistically calculated values 3E
Paratyphoid B	7		2	2.5	3		
Paratyphoid A	1		1--2	2--3	2--3	3--4	Absolute values 3E
Flexner's dysentery	1		1--1.6	1.2--2	2--3	2.3--4.5	